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Supercritical angle fluorescence (SAF) immunoassay platform

Thomas Ruckstuhl, Christian Winterflood, Stefan Seeger¹

Physikalisch-Chemisches Institut, Universität Zürich, Winterthurerstr. 190, CH-8057 Zürich,
Switzerland.

s.seeger@pci.uzh.ch

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An inexpensive and easy-to-use immunoassay platform for the sensitive detection of analytes is presented. It comprises single-use polymer test tubes and a compact fluorescence reader. The optics for the capture of supercritical angle fluorescence (SAF) has been built into the tubes allowing for the extremely sensitive readout of solid phase immunoassays in real time and without washing steps. One-step sandwich immunoassays with interleukin 2 (IL-2) were carried out with capture antibodies immobilized in the tubes. At a turn around time of 12 minutes the limit of detection for IL-2 was 0.27 pM (4.5 pg/ml) and the linear range covered 3 orders of magnitude. The developed technology is also adaptable to well plates and has great potential of replacing the work-intensive and time-consuming enzyme-linked immunosorbant assay (ELISA).

KEYWORDS Supercritical angle fluorescence, polymer test tube, one-step immunoassay, sandwich test

¹ Corresponding author. Phone: +41 44 635 4450. E-mail: s.seeger@pci.uzh.ch.

INTRODUCTION

The immunoassay is a key technology for diagnostics in immunology, biochemistry and medicine [1]. To achieve high detection sensitivities heterogeneous assay formats are used, where analyte molecules accumulate on a solid substrate. Current immunoassay technologies are based on magnetic micro- and nanoparticles [2-6], surface plasmon resonance [7,8], surface enhanced Raman scattering [9], electrical detection [10], electro-chemiluminescence [11,12] and fluorescence detection [13-15]. The need for sensitive and cost-effective concentration measurements is driving the field of surface enhanced fluorescence, comprising various strategies to increase the fluorescence detection efficiency at surfaces [16]. Many of the developed concepts achieve a high sensitivity but also involve a high level of technical complexity, hampering their implementation in sensitive immunoassay systems affordable for a broad range of users such as in medical point of care diagnostics. Better solutions have been anticipated for a long time since the established detection technology, in particular the enzyme linked immunosorbant assay (ELISA), is lavish in the amount of work, material and time required.

Here, we introduce a fluorescence-based immunoassay platform for one step sandwich immunoassays within minutes. The system comprises single-use polymer test tubes with incorporated fluorescence collection optics for single use and a compact fluorescence reader. The platform is based on the detection of supercritical angle fluorescence (SAF) – a straightforward technology to monitor surface reactions in realtime [17-23]. SAF occurs for fluorescent molecules located at the surface of a transparent substrate and is emitted above the critical angle of total internal reflection into the substrate. The critical angle of the interface between aqueous sample (refractive index: $n_1 = 1.33$) and the substrate ($n_2 = 1.52$) is $\theta_c = \arcsin(n_1/n_2) \approx 61^\circ$. SAF is a consequence of the impact of the boundary on an emitter's near field. For molecules located directly at the surface SAF constitutes more than one third of the overall emission. As shown in Figure 1, the near-field coupling decays exponentially with the distance to the boundary [24,25]. A straightforward parabolic element can convert SAF into conveniently detectable parallel rays by loss-free total internal reflection (TIR). This strategy combines high collection efficiency with surface-selective detection and is therefore useful for the sensitive

readout of immunoassays. The outstanding signal-to-noise ratio of the SAF technology has been used for single molecule detection [26], fluorescence microscopy [27] and fluorescence correlation spectroscopy [28].

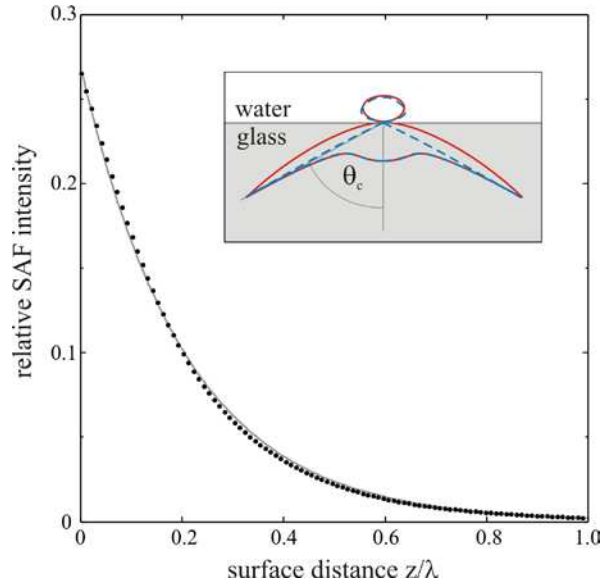


Figure 1 SAF emission at a water/glass interface. The proportion of fluorescence emitted into the supercritical angles between 63° and 78° was calculated for isotropically orientated fluorophores according to Ref. [24] (points). The dependency of the SAF intensity on the surface distance can be approximated by a mono-exponential with a decay constant of 0.2λ (solid line). The inset shows polar plots of the angular distribution of radiation for fluorescence emitters with surface distances of $z = 0$ (solid) and $z = \lambda/3$ (dashed).

Due to the straightforward detection optics it can be produced by polymer injection molding at low cost. In the following sections we report on the integration of the detection optics into polymer test tubes and the development of a fluorescence readout instrument. The system is tested by performing immunoassays with interleukin 2 (IL-2) in a one step sandwich test format. IL-2 is a cytokine of

15.5 kD, produced by T lymphocytes when stimulated by microbial infection. In medical diagnostics, for example, IL-2 serves as a marker for mycosis.

EXPERIMENTAL SECTION

Disposable test tube. The tube is composed of two polymer parts of customized design and a standard o-ring with an outer diameter of 6 mm (Figure 2). The lower part containing the optics, referred to as substrate, was fabricated by injection molding of Zeonex (Zeon Chemicals, Japan), a cyclo-olefin polymer. An aspheric surface at the bottom side serves to focus the laser excitation onto the upper surface of the substrate. The asphere has a focal length of 5.6 mm and is designed to produce a homogeneous light disc of 50 μm diameter when fed with a collimated excitation beam of 1.5 mm diameter and uniform intensity profile. The parabolic surface has an outer diameter of 8 mm and a focal length of 1.2 mm. Air surrounds the parabolic surface resulting in total internal reflection (TIR) of the fluorescence generated at its focal point. The parabolic focal point is located at the center of the upper flat surface which constitutes the interface to the analyte solution. SAF emitted at surface angles between 63° and 78° is collimated by TIR and exits the tube at the bottom side through the flat surface surrounding the asphere. The cylindrical base of the substrate is the connector between tube and fluorescence reader. The optical interfaces (asphere, bottom flat) are concealed inside this hollow cylinder to prevent damage and contamination. The substrates were fabricated with high optical accuracy by Syntec Optics (Pavillion, NY, USA).

The black part with lid was injection molded from polycarbonate and black additive. A snug fit establishes a strong junction between the tube parts and without the use of adhesives. In combination with the o-ring the tube is sealed hermetically. The lid provides an airtight seal. The tube has the same diameter (10.7 mm) as its well-known counterpart from Eppendorf and is therefore compatible with common laboratory equipment (shakers, centrifuges, thermocylers, etc.).

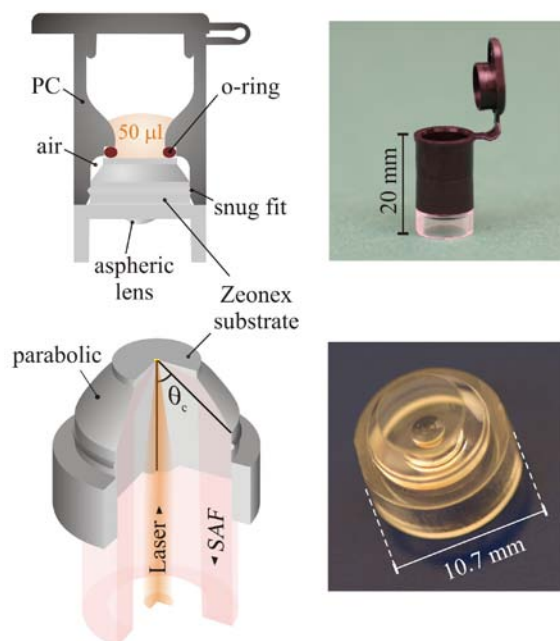


Figure 2 Schematics and photographs of the test tube and the optical substrate.

Fluorescence reader. For the fluorescence readout the tube needs to be provided with a collimated beam and returns a collimated ring of SAF. The instrument, equipped with a laser diode (635 nm, HL6335G, Hitachi, Japan) and photomultiplier module (H8259-02, Hamamatsu, Japan), is shown in Figure 3. The laser diode is temperature stabilized at 32°C. Pre-commissioning the laser beam is aligned by two mirror beam steering and its diameter is reduced to 1.5 mm by means of a circular aperture. The intensity profile of the laser beam across the small aperture is uniform within 15% which leads to a high homogeneity of the excitation intensity at the sensing region.

A motorized filter wheel is used to swivel a neutral density filter to switch between the excitation intensities of 1 μ W and 1 mW. A small reflection prism (NT45-386, Edmund optics, USA) is used to separate the optical paths of fluorescence excitation and detection. Collimated fluorescence from the tube is refocused with a lens ($f = 100$ mm) through a circular detection aperture of 3 mm diameter, 100 mm behind the lens. Interference filters are used in the excitation path (BrightLine HC 632/22, Semrock, USA) and in front of the detector (BrightLine HC 676/29, Semrock). A cylindrical cavity on top of the device serves as an adapter for the tubes. Due to the design of the substrate optics the

excitation focus is positioned exactly at the center of the substrate just by insertion of the tube into the adapter. The prototype reader is built into a plastic casing of 254 mm × 180 mm × 90 mm. Data acquisition, actuation and control was done using incorporated electronic circuits and a laptop via USB.

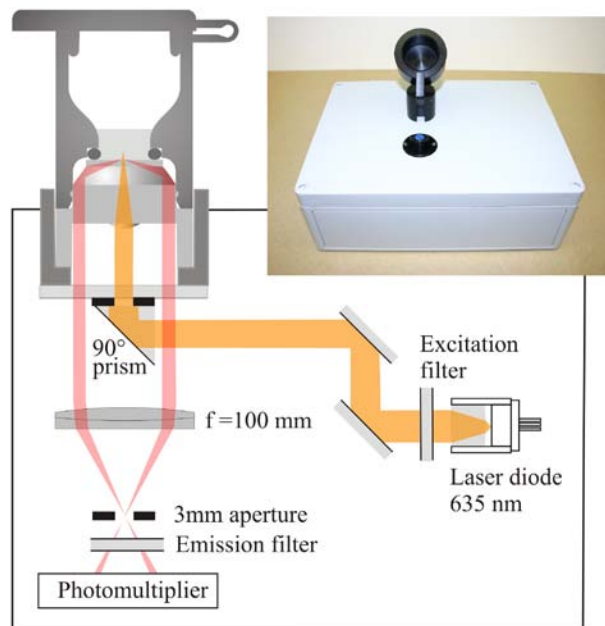


Figure 2 Schematic and photograph of the fluorescence reader prototype.

Surface chemistry. Antibody immobilization on Zeonex was done as described in Ref. [15]. In brief, before assembly of the tubes, the Zeonex substrates were activated by oxygen plasma (40 kHz/100 W/0.2-1 mbar) for 5 min and silanized by immersion in a 3% (v/v) solution of 3-aminopropyl triethoxysilane in ethanol for 2 h. The tubes were rinsed with ethanol and water, dried under nitrogen flow and left to cure over night. The silanized Zeonex was functionalized with aldehyde-activated dextran by Schiff's base coupling. For this it was immersed in a solution of 2% (w/v) dextran T40 and 30 mM sodium periodate for 2 h, rinsed with ddH₂O and further oxidized in 30 mM sodium periodate for 2 h. The Zeonex substrates were assembled with the o-ring and the upper tube part. Streptavidin was immobilized by Schiff's base coupling by filling the tubes with 50 µl of a 1 mg/ml solution in phosphate buffered saline (0.01 M PBS, pH 7.4) and incubating them over night at 4°C. The

tubes were further incubated with 100 μ l of 5 mM glycine/PBS to block unreacted aldehydes. The tubes were washed several times with 0.05% (v/v) Tween20 in PBS. For later use the tubes were incubated with 100 μ l of Liquid Plate Sealer® (Candor Bioscience) for 1 h at 4°C. The solution was removed and the tubes were dried under nitrogen flow. After this treatment the tubes can be stored at 4°C in dry conditions for a longer period. For the presented experiments the storage time was within 2 weeks. For tubes that were stored for 5 months before the immobilization of capture antibodies the SAF intensity increase measured during the immunoreactions was reduced by about 20%.

Prior to the assays, the tubes were incubated for 1 h with 50 μ l of the biotinylated monoclonal capture antibody rat anti-mouse IL-2, clone JES6-5H4 (eBioscience), at 30 μ g/ml in PBS and washed several times with 0.05% (v/v) Tween20 in PBS. The tubes were blocked for 1 h with 3% (w/v) bovine serum albumin/0.05% (v/v) Tween20 in PBS. Recombinant mouse IL-2 (Invitrogen) was used as the analyte and Cy5-labeled monoclonal rat anti-mouse IL-2, clone JES6-1A12, was used as the detection antibody. All assays were performed with a total of 50 μ l and with a final concentration of 1.5 nM of detection antibody. The assays were performed in 3% (w/v) bovine serum albumin/0.05% (v/v) Tween20 in PBS.

Polymer autofluorescence reduction. With regards to autofluorescence, optical polymers are inferior to high-grade optical glasses. A useful strategy to minimize the background contribution from the substrate is to photobleach the autofluorescence by light exposure of the polymer before the measurement [29, 30]. This was done by illuminating the tubes from below with 630 nm LED light (ELJ-630-211, Roithner, Vienna, Austria) for 1 h at 4°C. The light was focused by the aspheric and parabolic surface producing an irradiance in the order of 100 W/cm² at the detection region of the tube.

RESULTS AND DISCUSSION

The developed system features an excellent fluorescence collection efficiency of 30%, which is comparable to the signal strength of microscope objectives with numerical aperture as high as 1.2. For the sensitive fluorescence detection on plastic substrates a problem to be solved is the inherent polymer

autofluorescence contributing to the background of the measurement. The exposure of the tubes to a high brightness LED reduced the background of the measurement by about 50% as shown in Figure 4.

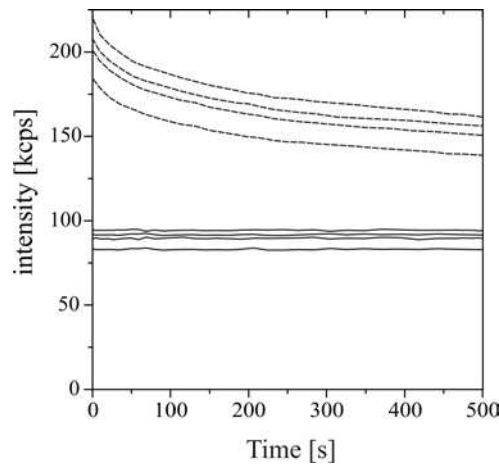


Figure 4 Background intensity of 8 tubes measured on the prototyped fluorescence reader with (solid) and without (dashed) prior exposure of the tubes to LED light for 24 h.

Photobleaching of the polymer alone was not sufficient to accomplish a sensitivity below picomolar. The key strategy was to minimize the detection volume inside the substrate using a spatial filter. The design goal for the detection volume was to minimize the collected volume element for light scattering and autofluorescence inside the polymer substrate without interfering with the SAF collection from the excited surface area of 50 μm diameter. To this end, the parallel ring of SAF delivered by the substrate optics was spatially filtered by the reader optics. According to raytracing calculations, a lens of focal length $f = 100$ mm focusing SAF through a circular aperture of 3 mm diameter reduces the penetration of the collected volume inside the substrate to about 70 μm (Figure 5A). An important requisite for the successful implementation of this strategy was the high optical accuracy of the single-use polymer substrate. The substrates were subjected to extensive optical tests showing excellent accuracy within tolerances. For example, the aspheric surface has an irregularity of only two fringes and the geometrical focus of the parabolic surface is located at the surface of the substrate within an accuracy of 5 μm . The accuracy of the laser excitation spot was confirmed by the dark spot photobleached into a homogeneous

fluorescent coating. A substrate coated with Cy5-labelled antibodies was illuminated on the reader device with an intensity of 1 mW. The irradiance within the excitation disc was 50 W/cm^2 , sufficiently high to photobleach fluorescent molecules bound at the interface within a few seconds. Afterwards the substrate was inspected by fluorescence microscopy showing excellent agreement with the targeted spot diameter of $50 \mu\text{m}$ (Figure 5B). Using the strategy of spatial filtering the count rate obtained for blank substrates was $90 \pm 15 \text{ kcps}$ (kilo counts per second) for an excitation intensity of 1 mW.

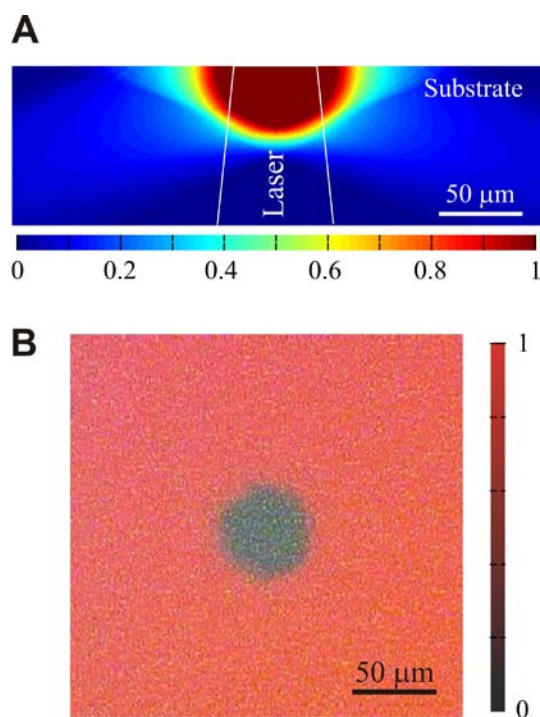


Figure 5 (A) Spatial collection efficiency function calculated by ray tracing. Spatial filtering reduces the extent of the collected volume element inside the substrate to less than $100 \mu\text{m}$. The marginal rays of the laser beam are also indicated. (B) Fluorescence image of a fluorescence coated substrate after photobleaching on the reader instrument. The diameter of photobleached area (dark) of $50 \mu\text{m}$ diameter corresponds to the laser excitation spot at the surface.

IL-2 assays were carried out in a sandwich test format with two antibodies of different site specificity. By using antibodies binding to spatially divided sites of the analyte the immunoreactions can be performed simultaneously without interfering with each other. A protocol was established that limits the maximum time need of the assay to about 13 minutes while minimizing the number of working steps. 45 μL of IL-2 dilutions in buffer were pipetted into the tube coated with capture antibodies. Immediately afterwards, 5 μL of a 15 nM detection antibody solution were added and mixed with a pipette resulting in a final detection antibody concentration of 1.5 nM. The tube was then inserted into the reader instrument and the measurement was started. The measurement was controlled by dedicated software from a laptop and required no further working steps from the operator. Due to the high surface selectivity of the SAF method washing steps could be omitted in the assay protocol. During the first 700 s the formation of the sandwich complexes at the surface was monitored by measuring the increase of the SAF intensity with time. An excitation intensity of 1 μW was used producing a low irradiance of 0.05 W/cm^2 within the illuminated spot at the surface. The photobleaching of surface bound fluorophores was kept well below 1% by measuring the SAF intensity only from time to time with a laser exposure time of 1 s. Measurements of selected IL-2 concentrations are shown in Figure 6A. The high signal to noise ratio of the system makes it possible to obtain smooth binding curves from picomolar analyte concentrations. The binding kinetics was rather complex as the sandwich formation at the surface had two pathways with IL-2 molecules binding to either the detection antibody or the capture antibody first. In the calibration curve shown in Figure 6B the intensity obtained after 700 s is plotted versus the concentration for IL-2 concentrations ranging from 30 pM and 5 nM. Data points for concentrations up to 300 pM were fitted with a straight line through the origin.

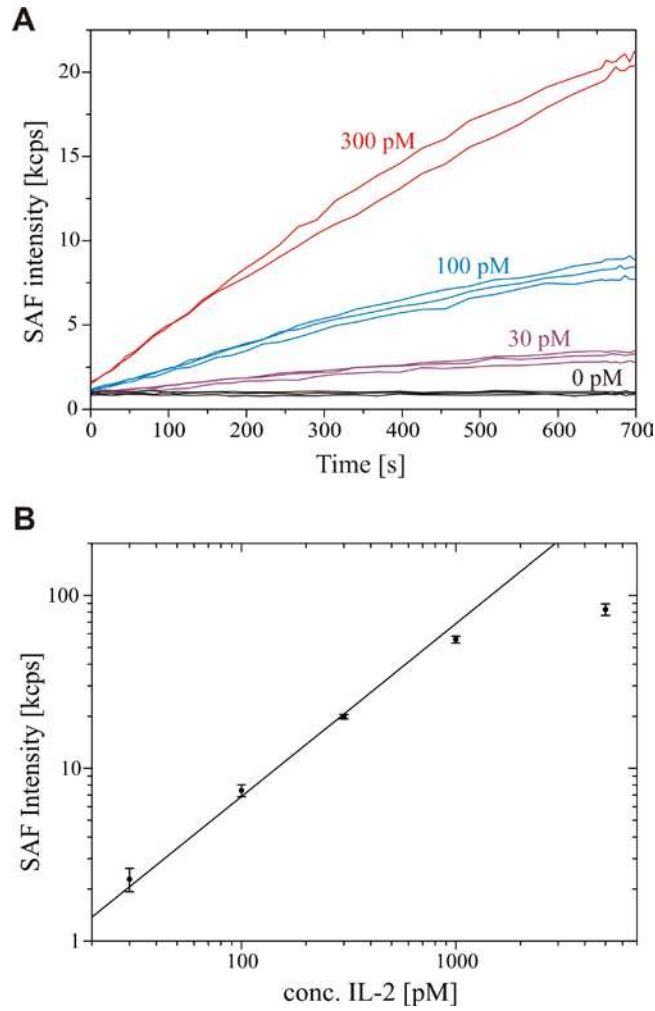


Figure 6 (A) Realtime measurements for different IL-2 concentrations. (B) Increase of the SAF intensity after 700 s plotted against IL-2 concentration. A straight line through the origin was fitted to the data for IL-2 concentrations below 1 nM. At IL-2 concentrations higher than 1 nM the linear relationship is lost, due to depletion of the free detection antibodies.

The SAF intensity approaches a saturation level for IL-2 concentrations above 1 nM as a result of the depletion of the free detection antibodies. With 1.5 nM, a rather low concentration was chosen to optimize the conditions for the detection of very low analyte concentrations. By using higher concentrations of the detection antibodies the dynamic range of the IL-2 measurement can be extended by almost two orders of magnitude until depletion of the free capture antibodies will come into play.

When measuring very low analyte concentrations it was necessary to use a higher excitation intensity to enhance the detectable fluorescence signal. This however led to an increased photobleaching rate of the fluorophores, which is a welcome effect for our approach. The amplitude of the intensity decay obtained after extensive photobleaching of the surface-bound fluorophores serves as a precise measure for the amount of fluorescent analyte present at a surface [21,26]. The background contribution of the measurement substrate can vary from one consumable to the next. For sensitive measurements it is usually necessary to perform blank measurements and subtract the background intensity from the fluorescence signal. For routine use, however, such procedure is unfavorable as it increases the amount of work for the user or the complexity of automated assay systems. This issue is circumvented by the photobleaching method where the intensity decays are unaffected by the photostable background of the substrate. This method was applied in the IL-2 assay by increasing the excitation intensity to 1 mW, 700 s after the start of the measurement. The photobleaching decays for a range of concentrations are shown in Figure 7A. After an exposure time of only 11 s, over 95% of the surface-bound fluorescence was bleached. The intensity decay observed in absence of analytes was caused by photobleaching of the detection antibodies that adsorbed non-specifically on the substrate. The average amplitude of the zero concentration measurement was later subtracted from the decay amplitudes of all IL-2 concentrations. The resulting background corrected decay amplitude plotted against the IL-2 concentration is shown in Figure 7B. The data was fitted by a straight line through the origin. The limit of detection was calculated from its intersection with the 3σ value of the zero concentration measurements to be 0.27 pM (4.5 pg/ml).

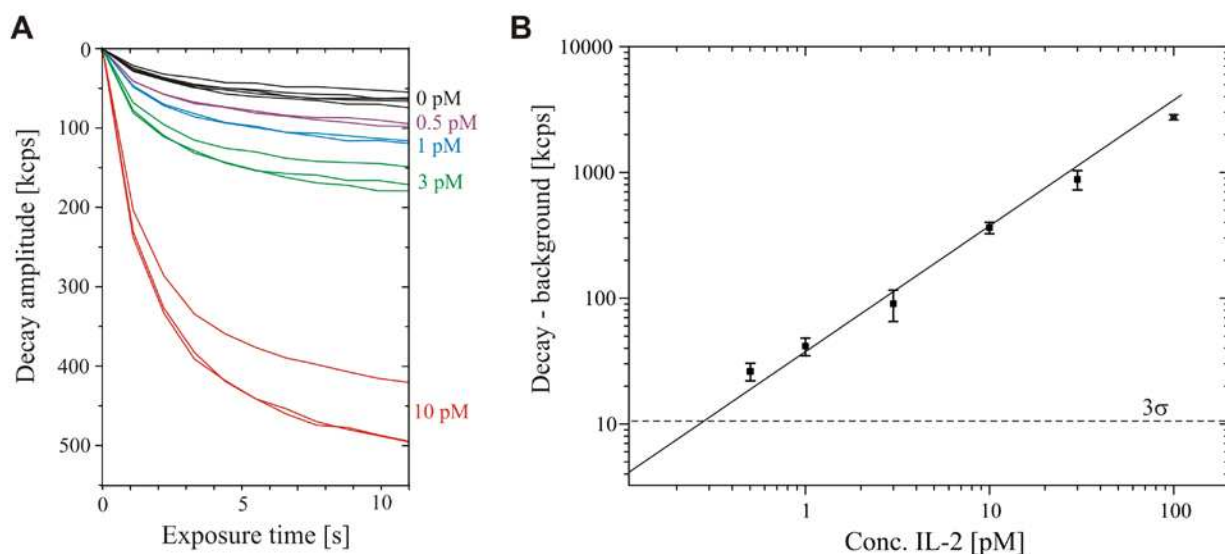


Figure 7 Sensitive readout after 700s with 1 mW excitation. (A) Photobleaching decays of the SAF intensity. (B) Plot of the photobleaching amplitudes after 11s minus background (zero concentration decay) against IL-2 concentration. A straight line through the origin was fitted to the data for IL-2 concentrations up to 30 pM. The maximum count rate processable by the electronics was 7 Mcps.

According to the supplier of the employed antibodies (eBioscience) their recombinant standard range in a sandwich ELISA is 4 pg/ml – 500 pg/ml. The duration for this widely used test is approximately 4 hours (excluding the time needed to immobilize the capture antibodies) and involves numerous incubation and washing steps. In comparison, the presented assay platform provides a linear response for a concentration range of 4.5 pg – 10 ng/ml. The duration of the assay was 13 minutes and involved just two consecutive liquid additions. It should be straightforward to reduce this to one addition by depositing lyophilized detection antibodies inside the tube at the production process. The coefficient of variation (CV) of the assay was 12.4% averaged over all measurements. The main contribution of this CV was traced back to the variation of the density of the antibody coating from one tube to another, resulting from the manual immobilization procedure.

We emphasize that the obtained sensitivity was achieved without any improvement of the mass transport towards the surface. During the immunoassay the high surface density of capture antibodies caused a depletion of the analyte concentration in the surface-near region and the binding rate of analytes was limited by diffusion from remote regions. Consequently, it is possible to further increase the assay sensitivity by enhancing the mass transport. The assay was repeated 4 times with an IL-2 concentration of 100 pM at which the tubes were agitated by an orbital shaker (Vibrax VXR, IKA, Germany). After 700 s the SAF intensity was measured with 1 μ W excitation intensity and with 186 ± 19 kcps about 2.5-fold higher than obtained for the same assay in static condition.

OUTLOOK AND CONCLUSION

Further improvements can be expected from ongoing work to restrict the coating of the capture antibody to the region of the detection volume in the center. For simplicity of the immobilization procedure the coating was applied across the entire contact area of the substrate with the analyte solution of 4 mm diameter. Only about 0.02% of the IL-2 molecules which were captured on the substrate bound within the detected area.

The proprietary technology for the capture of SAF in single use receptacles is suitable for integration into well plates [31]. The optical arrangement tolerates a substantial lateral mismatch in the order several hundred micrometers between its symmetry axis and the excitation beam and rapid sequential readout of numerous wells can be achieved using inexpensive actuators (Figure 8). For comparison, with the focusing element included in the reader instead of the receptacle the lateral tolerance of the alignment is only few ten micrometers [17].

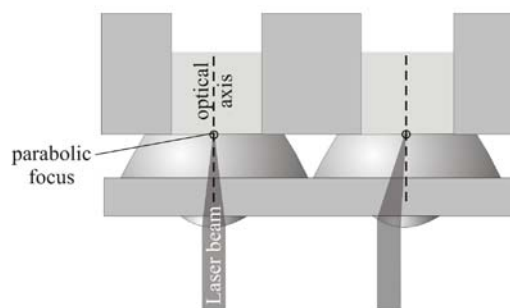


Figure 8 The detection optics built into the tube is also suited for the integration into well plates. The design is extremely tolerant regarding the lateral alignment of the laser beam allowing for rapid sequential readout of the wells.

On the basis of the distinguished SAF technology an immunoassay platform has been developed that is on the one hand very easy-to-use and robust, on the other hand achieves extreme assay sensitivity. The SAF immunoassay platform addresses the need for fast and sensitive concentration measurements at low cost and could be useful for all bioanalytical fields.

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